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# Appendix II

# *In vitro* effects of estetrol on receptor binding, drug targets and human liver cell metabolism

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## INTRODUCTION

In this Appendix, data are reported obtained by *in vitro* studies, performed with the human fetal steroid estetrol ($E_4$). The studies have been executed by a commercial laboratory in Taiwan (MDS). Estetrol was synthesized by Syncom, Groningen, The Netherlands. Estetrol was >98% pure and did not contain a detectable level of 17$\beta$-estradiol ($E_2$), as determined by high-power liquid chromatography/mass spectrometry, nuclear magnetic resonance and DSC analysis (results not shown).

Data from the following studies are reported in this Appendix:

- Receptor binding studies
  - Estrogen receptors (ER$\alpha$ and ER$\beta$)
  - Other steroid receptors (glucocorticoid, progesterone and testosterone)
  - Other receptors and molecular targets ($n = 124$)
- Metabolism in human and rat hepatocytes
- Inhibition of P450 enzymes

For a discussion on the relevance of these data, the reader is referred to the other papers in this Supplement.

## RECEPTOR BINDING

### Estrogen receptors

The affinity of $E_4$ at the ER$\alpha$ and ER$\beta$ estrogen receptor has been studied in human recombinant insect Sf9 cells, using [$^3$H]estradiol as ligand. Estetrol showed an $IC_{50}$ value of $17 \pm 2$ nmol/l, corresponding to a $K_i$ value of $4.9 \pm 0.567$ nmol/l, at the ER$\alpha$ receptor and an $IC_{50}$ value of $91 \pm 3$ nmol/l, corresponding to a $K_i$ value of $19 \pm 1$ nmol/l, at the ER$\beta$ receptor. The Hill coefficients were close to 1, both for the ER$\alpha$ receptor ($1.01 \pm 0.136$) and the ER$\beta$ receptor ($0.992 \pm 0.0323$), indicating a single binding site interaction. Thus, $E_4$ has a four to five times higher affinity to the ER$\alpha$ receptor compared to the ER$\beta$ receptor.

The non-selective estrogen diethylstilbestrol (DES), used as a reference compound, had higher affinities at the ER$\alpha$ receptor and the ER$\beta$ receptor compared to those found with $E_4$, with $K_i$ values of $0.286 \pm 0.111$ nmol/l and $0.199 \pm 0.049$ nmol/l, respectively (Figures 1 and 2).

### Other steroid receptors

To determine the selectivity of $E_4$ for the estrogen receptor compared to other steroid receptors, the binding of $E_4$ at the glucocorticoid, progesterone and testosterone receptors was studied. Estetrol, at a prime concentration of 10 $\mu$mol/l, hardly inhibited the dexamethasone binding at the human glucocorticoid receptor in human Hela 53 cells (15%), the R-5020 binding at the bovine uterus progesterone receptor (11%) or the mibolerone binding at the rat recombinant testosterone receptor in *Escherichia coli* (11%).

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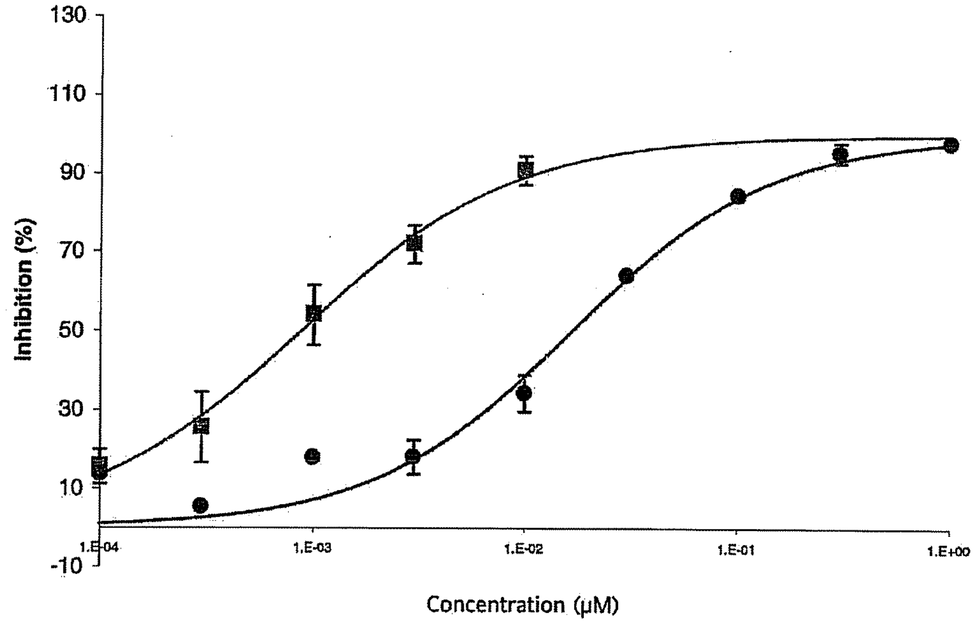


**Figure 1** Binding of estetrol (circles) and diethylstilbestrol (squares) at ERα receptors

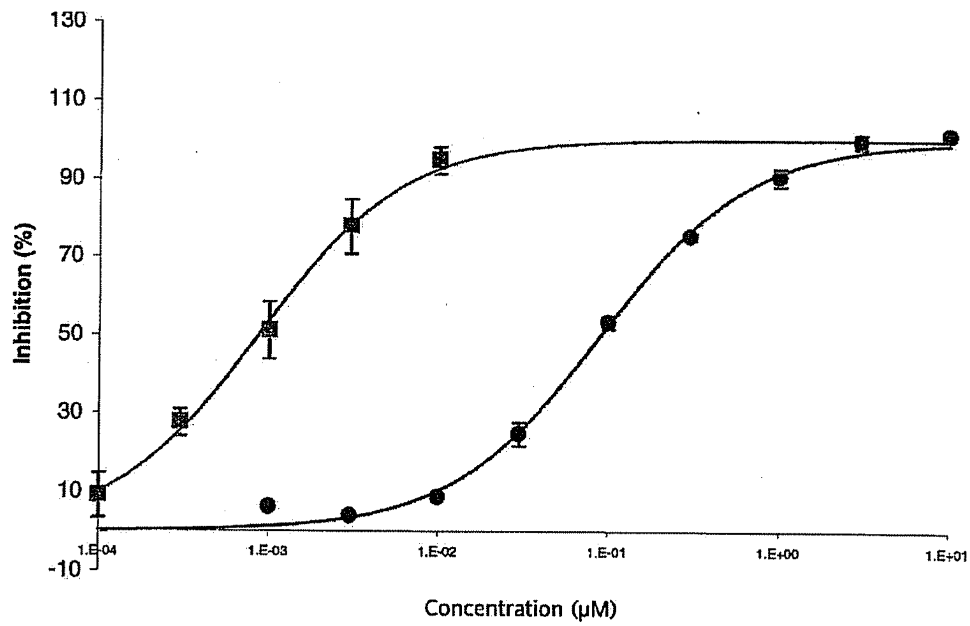


**Figure 2** Binding of estetrol (circles) and diethylstilbestrol (squares) at ERβ receptors

## Other receptors and enzymes

Potential interactions of $E_4$ with 124 human non-steroidal receptors and enzymes belonging to various classes of drug targets were investigated in radioligand binding assays.

For each assay, methods were adopted from the literature. Estetrol was assayed at a final concentration of $10^{-5}$ mol/l for inhibitory effects and, for each assay, reference standards were run in parallel to validate the results obtained. At the

tested concentration of $10^{-5}$ mol/l, $E_4$ did not relevantly inhibit any of the following drug targets for binding their respective radiolabeled ligands: adenosine $A_1$, $A_{2A}$, $A_{2B}$, $A_3$; adenosine transporter; adrenergic receptor $\alpha_{1A}$, $\alpha_{1B}$, $\alpha_{1D}$, $\alpha_{2A}$, $\alpha_{2B}$, $\alpha_{2C}$, $\beta_1$, $\beta_2$, $\beta_3$; adrenergic norepinephrine transporter; angiotensin $AT_1$, $AT_2$; atrial natriuretic factor; bombesin; bradykinin $B_1$, $B_2$; calcitonin; calcitonin gene-related peptide; calcium channel type L; calcium channel type N; cannabinoid $CB_1$; cannabinoid $CB_2$; chemokine CCR1; chemokine CCR2B; chemokine CCR4; chemokine CCR5; chemokine CXCR1; chemokine CXCR2; cholecystokinin $CCK_A$; cholecystokinin $CCK_B$; choline transporter; dopamine $D_1$; dopamine $D_{2L}$; dopamine $D_3$; dopamine $D_{4.2}$; dopamine $D_5$; dopamine transporter; endothelin $ET_A$; endothelin $ET_B$; epidermal growth factor; GABA transporter; $GABA_A$; $GABA_B$; galanin GalR1; galanin GalR2; glucagon-like peptide-1; glutamate receptor; glycine; histamine receptor $H_1$; histamine receptor $H_2$; histamine receptor $H_3$; imidazoline $I_2$; insulin; interleukin IL-1$\alpha$; interleukin IL-2; interleukin IL-6; leukotriene $B_4$; leukotriene $D_4$; melanocortin MC3; melanocortin MC4; melanocortin MC5; melatonin ML1; muscarinic receptor M1, M2, M3, M4, M5; neuropeptide Y1, Y2; neurotensin; nicotinic receptor; opiate receptor $\delta$, $\kappa$, $\mu$; orphanin ORL1; phorbol ester; platelet activating factor; platelet-derived growth factor; potassium channel $K_A$, $K_{ATP}$, $K_V$, $SK_{CA}$; prostanoid $EP_1$, $EP_4$; purinergic receptor $P_{2X}$, $P_{2Y}$; serotonin 5-$HT_{1A}$, 5-$HT_{1B}$, 5-$HT_{2A}$, 5-$HT_{2B}$, 5-$HT_{2C}$, 5-$HT_3$, 5-$HT_4$, 5-$HT_{5A}$, 5-$HT_6$, 5-$HT_7$; serotonin transporter; sigma $\sigma_1$, $\sigma_2$; sodium channel site 2; tachykinin $NK_1$, $NK_2$; $NK_3$; thromboxane $A_2$; thyrotropin releasing hormone; transforming growth factor $\beta$; tumor necrosis factor; vascular endothelial growth factor; vasoactive intestinal peptide $VIP_1$; or vasopressin $V_{1A}$.

Estetrol, tested at a prime concentration of 10 $\mu$mol/l, did not show inhibition of the binding of the respective ligands larger than 20% in 123 of the 124 assays studied.

Estetrol inhibited the binding of prazosin at the adrenergic $\alpha_{1B}$ receptor by 23% at a concentration of 10 $\mu$mol/l.

## *IN VITRO* METABOLISM

The *in vitro* metabolic stability and the occurrence of $E_4$ metabolites were studied in freshly prepared female rat hepatocytes and in cryopreserved female human hepatocytes.

Estetrol and metabolites were detected, characterized and profiled using liquid chromatography/mass spectrometry analysis.

### Metabolic stability

Estetrol was moderately susceptible to *in vitro* metabolism in both rat and human hepatocyte incubations (Figure 3). After a 4-h incubation period of 1 $\mu$mol/l $E_4$ with rat hepatocytes, 48% of $E_4$ was unchanged. After incubation with 10 $\mu$mol/l $E_4$, 30% of $E_4$ was still present. After incubation for the same 4-h period with human hepatocytes, 27% of $E_4$ was left with the lower dose of $E_4$ (1 $\mu$mol/l) and 49% was unchanged with the higher dose (10 $\mu$mol/l). So, in contrast to the rat hepatocytes, a higher percentage of unchanged $E_4$ was found in human hepatocytes with increased substrate concentration. This observation indicates that the metabolism of $E_4$ in human

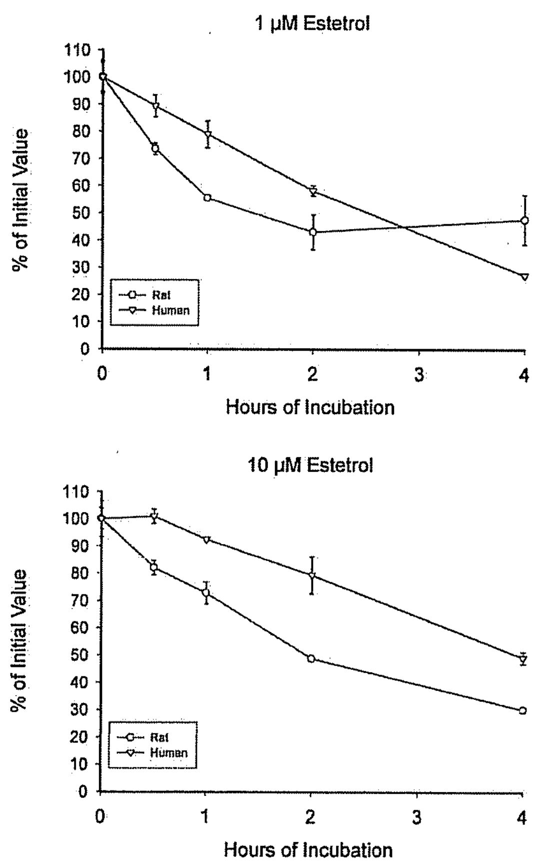


**Figure 3** Disappearance of estetrol following incubation with rat and human hepatocytes

hepatocytes was partially saturated at the concentration of 10 μmol/l.

The rates of $E_4$ metabolism in human hepatocytes were 0.5 and 3.8 nanomoles/h/$10^6$ cells at 1 and 10 μmol/l substrate concentrations, respectively. In rat hepatocytes, corresponding rates of metabolism were 1.1 and 6.7 nanomoles/h/$10^6$ cells. Thus, human hepatocytes have a lower metabolic rate compared to rat hepatocytes.

### Metabolites

Estetrol was metabolized into at least five metabolites (M1 to M5; Table 1). Metabolites were attentively identified based on observed mass-to-charge (*m/z*) values, retention times and the known structure of the parent compound. The metabolite profile was fully species-dependent. Rat hepatocytes produced the metabolites M1 and M2, but not M3, M4 or M5, and human hepatocytes produced M3, M4 and M5, but not M1 and M2. Metabolite M3 appeared only in small quantities and only after 4-h incubation at the 10 μmol/l $E_4$ concentration. The ratio of metabolites changed markedly over the concentration range examined. The predominant metabolite in the 1 μmol/l $E_4$ incubation was M4, which accounted for 75% of the metabolites. The major metabolite in incubations with 10 μmol/l $E_4$, however, was M5, representing 77% of all metabolic products. From the *m/z* spectra, the retention times and the known chemical structure of $E_4$, it could be deduced that the metabolites were formed via methylation, hydroxylation, dehydroxylation, glucuronidation and sulfation. However, alternative pathways that could not be ruled out include dehydrogenation, dihydroxylation and disulfation. A summary of the metabolites and the proposed metabolic pathways involved is provided in Table 1.

## INHIBITION OF P450 ENZYMES

The effects of $E_4$, $E_2$ and ethinylestradiol on P450 enzyme activity were examined using the Baculovirus insect cell line Sf9 that expresses the recombinant human P450 enzymes CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4. The data are summarized in Table 2.

Estetrol, at a concentration of 10 μmol/l, had no inhibitory effects on any of the P450 enzymes studied. In contrast, $E_2$, at a concentration of 10 μmol/l, moderately inhibited CYP1A2 by 19%, and strongly inhibited CYP2C19 by 63%.

**Table 1** Metabolites of estetrol in rat and human hepatocytes as detected by liquid chromatography/mass spectrometry

| *Analyte* | $t_R$ (min)* | $Rt_R$† | *M/z*‡ | *Mass change* | *Proposed biotransformation* | *Rat* | *Human* |
|---|---|---|---|---|---|---|---|
| Estetrol | 8.74 | 1.00 | 303 | – | parent | | |
| M1 | 9.51 | 1.09 | 333 | +30 | methylation + hydroxylation | +** | – |
| M2 | 9.12 | 1.06 | 301 | −2 | methylation + dehydroxylation | + | – |
| M3 | 6.19 | 0.69 | 461 | +158 | dehydroxylation + glucuronidation | – | + |
| M4 | 4.73 | 0.54 | 383 | +80 | sulfation | – | + |
| M5 | 3.86 | 0.44 | 479 | +176 | glucuronidation | – | + |

*, Average HPLC retention time; †, average retention time relative to estetrol; ‡, mass-to-charge ratio; ** +, detected; –, not detected

**Table 2** Effects of estetrol, estradiol and ethinylestradiol on P450 enzymes

| | *Inhibition (% sample solvent control)* | | | | |
|---|---|---|---|---|---|
| *Sample* | *CYP1A2* | *CYP2C9* | *CYP2C19* | *CYP2D6* | *CYP3A4* |
| Estetrol | 9 | 4 | 6 | −2* | −23 |
| Estradiol | 19 | −60 | 63 | 0 | −83 |
| Ethinylestradiol | 7 | −28 | 82 | 5 | 45 |

*, Negative values denote activation

Ethinylestradiol moderately inhibited CYP3A4 and strongly inhibited CYP2C19. The strong inhibition of CYP2C19 by $E_2$ and ethinylestradiol was comparable to the reference compound tranylcypromine, that induced a 61% inhibition at a concentration of 10 $\mu$mol/l.

Estradiol and ethinylestradiol activated CYP2C9 by 60 and 28%, respectively and $E_4$ and $E_2$ induced activation of CYP3A4 by 23 and 83%, respectively.

## CONCLUSIONS

Estetrol has a moderate affinity for human ER$\alpha$ and ER$\beta$ receptors with $K_i$ values of $4.9 \pm 0.567$ nmol/l and $19 \pm 1$ nmol/l, respectively, demonstrating a four- to five-fold preference for the ER$\alpha$ (lower $K_i$ value).

Estetrol has excellent selectivity for the estrogen receptors. Binding at the glucocorticoid, progesterone and testosterone receptors was only 11–15% at a concentration of 10 $\mu$mol/l and further profiling of $E_4$ in a set of 124 receptors and enzymes demonstrated inactivity towards 123 molecular targets. The single target showing interaction with $E_4$ was the adrenergic $\alpha_{1B}$ receptor (weak binding).

In rat and human hepatocytes, the rate of metabolism of $E_4$ was comparable and slow, complying with slow elimination *in vivo*.

The metabolites found after incubation of $E_4$ with rat and human hepatocytes were completely different. Metabolites produced by rat hepatocytes were not produced by human hepatocytes and *vice versa*. In rat hepatocytes, phase I metabolism is most important. This may result in active metabolites in the rat, whereas inactivation by glucuronidation and sulfation, i.e. phase II metabolism, are the pathways observed in human hepatocytes. This confirms that, in the human, $E_4$ is an end-stage product of metabolism and has no active metabolites.

Estetrol at a high concentration of 10 $\mu$mol/l did not inhibit the major cytochrome P450 enzymes CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4. Estradiol and ethinylestradiol significantly inhibited CYP2C19. Ethinylestradiol had a strong inhibitory effect on CYP3A4, whereas $E_2$ stimulated this enzyme significantly and $E_4$ had some stimulatory effect. These results suggest that $E_4$ may exhibit less interference with concomitantly administered drugs (drug–drug interaction) compared to ethinylestradiol and $E_2$.